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Published in:
Liver Transplantation

DOI:
10.1002/lt.25290

Publication date:
2018

Article Type: Letters from the Frontline

Production of Physiologically Relevant Quantities of Hemostatic Proteins During Normothermic Machine Perfusion of Human Livers

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Keywords
Coagulation, fibrinolysis, liver transplantation, normothermic machine perfusion, anticoagulation, plasma-free perfusion solution

Abbreviations

FFP – fresh frozen plasma

INR - international normalized ratio

NMP – normothermic machine perfusion

tPA – tissue-type plasminogen activator

VWF – Von Willebrand factor

Disclosure

The authors of this manuscript have no conflicts of interest to disclose. No financial support or grants were awarded to conduct this study.

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TO THE EDITOR

Ex-situ normothermic machine perfusion (NMP) of donor livers is an emerging innovation in liver transplantation that has made its transition to clinical trials. NMP is applied in an effort to overcome ischemia-reperfusion injury associated with static cold storage, to improve the quality of sub-optimally functioning donor organs and to permit viability testing of potentially transplantable livers. The *in-vivo* physiological conditions maintained during *ex-situ* NMP necessitate the need for a perfusion fluid that mimics the composition of whole blood. To date, perfusion fluids used in NMP studies are composed of either plasma-based solutions or plasma-free solutions consisting of colloids such as Gelofusine[1]. The latter avoids the use of human plasma which is scarce, costly and logistically challenging.

During *ex-situ* NMP, *in-vivo* graft reperfusion is mimicked and normal metabolic and synthetic functions are restored. It was previously demonstrated that the international normalized ratio (INR) of a plasma-free perfusion solution decreased over time during 8 hours of NMP of discarded human livers[2]. This suggests the production of procoagulant

proteins during NMP. In this study however, the levels of procoagulant proteins produced were not quantified and the INR values reported are likely an underestimation as the perfusion fluid contained substantial amounts of heparin, which is known to interfere with INR measurements. To date, duration of NMP of human donor livers in published data ranges from 3.5 to 24 hours. Bral et al. have even successfully transplanted a donor liver after 22.5 hours of NMP[3]. Based on published data, it is likely that physiological amounts of hemostatic proteins are produced during prolonged periods of NMP. Therefore, the objective of this study was to quantify the levels of individual hemostatic proteins during NMP of donor livers with a plasma-free perfusion solution.

Six human donor livers declined for transplantation by all three transplant centres in the Netherlands as well as by all other centres within the Eurotransplant region were included in this study. Five of the six livers were from DCD donors. The primary reasons for rejecting these livers for transplantation were; DCD livers from donors older than 60 years of age, ≥30% steatosis and uncontrolled cardiac arrest. Details on liver procurement, back-table and NMP procedures performed on these livers have been reported previously[4]. Livers were used for research purposes only, and were not intended to be transplanted following NMP. However, this research project has provided us important knowledge and insight in the ex-situ perfusion of livers using a blood product free perfusion fluid, which enabled us to start a clinical trial recently (www.trialregister.nl; #NTR5972)

The composition of the perfusion solution was described in detail previously[4]. In short, the livers were perfused with a 2202 ml heparinized solution containing Gelofusine, albumin, saline and the red blood cell substitute HBOC-201 (Hemopure®, HbO2 Therapeutics LCC. Souderton, PA, USA). Six plasma-based reference solutions using separate batches of fresh frozen plasma (FFP) provided to us by the blood bank were made in order to estimate the physiological relevance of the quantities of proteins generated in the plasma-free NMP perfusion solution. With exception of FFP, the composition and volume of this fluid was

identical to the plasma-free perfusion solution. These six different batches were used in order to establish a reference interval.

Concentrations of hemostatic proteins were analysed by enzyme-linked immunosorbent assays (ELISA), as the presence of heparin within the solutions prohibited meaningful quantification of these analytes using clot-based assays.

Prothrombin was quantified using the Human Prothrombin total antigen assay ELISA kit (Molecular innovations, Inc, Novi, MI, USA). Perfusate levels of FVII, FV, FX, plasminogen, and protein C were quantified using ELISA kits from Abcam (Cambridge, UK). Antithrombin levels were quantified using the Antithrombin Human-Serpin duoset ELISA (R&D systems; Bio-Techne, Oxon, UK), and tissue plasminogen activator (tPA) levels using the ZYMUTEST TPA-AG (Brenntag, Dordrecht, the Netherlands), respectively. All ELISAs were performed according to manufacturer's instructions. Von Willebrand factor (VWF) and fibrinogen antigen levels were determined with in-house ELISAs using commercially available polyclonal antibodies. (DAKO, Glostrup, Denmark).

The increase in hemostatic protein production during NMP is expressed in percentages. The median concentration for each protein at the end of NMP in the plasma-free perfusate is expressed as percentage of the median concentration of the same protein in the plasma-based reference solution. Correlation analysis was performed using SPSS version 23 for Windows (SPSS Inc., Chicago, Ill, USA).

Results and Discussion

Five of the six livers were from donation after circulatory death donors. The primary reasons for rejecting these livers for transplantation, donor demographics and the procedural variables of the six donor livers that underwent end-ischemic NMP have been described previously[4].

No hemostatic proteins were detectable in the plasma-free perfusion fluid samples prior to connection of the livers to the NMP device. During NMP, gradual increases in perfusate levels of all the proteins measured were observed in all livers (*Figure 1 A-J)*. Perfusate levels of procoagulant factors II, V, VII, X, and fibrinogen exhibited a gradual steady increase reaching levels of up to 10%, 9%, 59%, 9% and 31% of the plasma-based reference solution, respectively (*Supplementary Table 1)*. Substantial amounts of VWF were released by endothelial cells, reaching 76% of levels seen in the plasma-based reference solution. Levels of the anticoagulant proteins antithrombin III and protein C increased over time, with perfusate levels of protein C exceeding the levels in the plasma-based reference solution in some livers. Plasminogen gradually increased over time during NMP, amounting to close to 20% of normal plasma levels at the end of NMP. Perfusate levels of tPA peaked at 30 minutes and remained higher than tPA levels in the plasma-based reference solution. Interestingly, hemostatic protein production in the one DBD liver graft was not much different from the five DCD livers.

For most of the hemostatic proteins, we observed considerable variations in increase in concentrations in the perfusion fluid among the six livers (Figure 1). To assess whether these variations could be explained by variations in the recovery of hepatocellular function during NMP, we correlated hepatocyte-derived hemostatic proteins concentrations with markers of hepatic function, such as bile production and lactate concentration in perfusate. Indeed, most hemostatic protein concentrations in the perfusion fluid correlated well with bile production at the corresponding time points (Figure 2). Similar significant correlations were found between hemostatic proteins and lactate concentrations (*Supplementary Figure 1*). These results demonstrate that during 6 hours of *ex-situ* NMP, isolated donor livers perfused with a plasma-free solution are able to generate substantial amounts of hemostatic proteins. The production of procoagulant proteins ranged between 9-57% of the levels measured in the plasma-based reference solution. Anticoagulant and fibrinolytic protein levels amounted to 41-71% and 18-116% of the levels measured in the plasma-based reference solution,

respectively. In addition, appreciable amounts of the endothelial-derived proteins, VWF, and tPA released into the perfusate indicate that besides adequate hepatocyte synthetic activity, the secretory function of sinusoidal endothelium is also maintained during NMP.

To prevent coagulation activation and subsequent clot formation within the perfusion circuit and perhaps more importantly, in the liver itself, an anticoagulant agent is typically added to the perfusion solution during NMP. To date, anticoagulation management by most groups involves the use of heparin. Current NMP anticoagulation protocols in literature describe the addition of 10,000 to 25,000 units of heparin to the perfusion fluid during priming of the device. In some cases, additional boluses or continuous infusion are provided throughout NMP. Even though heparin is added to the machine perfusion fluid, heparin will only function as an anticoagulant in the presence of antithrombin. In this study, the concentrations of the procoagulant proteins (II, V and X) were generally lower compared to the anticoagulant and fibrinolytic proteins. Together with the profound production of tPA, this may indicate a favourable anticoagulant and profibrinolytic environment, at least in the first 6 hours of NMP. Additionally, antithrombin is generated in appreciable quantities and as the production of hemostatic proteins favours the anticoagulants, it is likely that heparin anticoagulation during plasma-free perfusate NMP is effective. Nevertheless, with production of all procoagulant proteins starting immediately after initiation of NMP, we postulate that more direct anticoagulants such as direct thrombin-inhibitors such as, Bivalirudin which, in contrast to other direct thrombin-inhibitors such as Dabigatran, does not require metabolic activation by the liver, may be more effective in the initial phase of NMP because such anticoagulants are antithrombin-independent.

Although limited by a small sample size, inclusion of only a single DBD liver and a relatively short perfusion period, this is to our knowledge, the first study to demonstrate the capability of donor livers to produce substantial amounts of hemostatic proteins during NMP using a plasma-free perfusion fluid.

Despite the liver being responsible for the synthesis of these hemostatic proteins, we have previously elucidated that plasma levels of hemostatic proteins are not controlled by the liver[5]. Hormonal systems, extra-hepatic sensors and clearance of these proteins were proposed as possible mechanisms responsible for the regulation of the plasma levels of hemostatic proteins. Given the absence of these influences during *ex-situ* NMP of an isolated liver, it is likely that the generation of these proteins will proceed throughout NMP. Particularly for studies where donor livers are perfused for extended periods of time (10+ hours), one can speculate that this could potentially lead to the generation of a perfusion solution with plasma-like or even supra-physiological concentrations of these proteins towards the end of NMP. Therefore, future research is required to further investigate whether current anticoagulant protocols using heparin are sufficient to prevent coagulation both in the very early and late phases of prolonged NMP.

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LEGENDS TO THE FIGURES

Figure 1. Increases in concentrations of pro-coagulant, anti-coagulant and fibrinolytic proteins in 6 human donor livers during 6 hours of normothermic machine perfusion (NMP) using a plasma-free solution. Panel A; Factor II. Panel B; Factor V. Panel C; Factor VII. Panel D; Factor X. Panel E; Fibrinogen. Panel F; Von Willebrand factor (VWF). Panel G; Protein C. Panel H; Antithrombin III. Panel I; Plasminogen. Panel J; Tissue-type plasminogen activator (tPA)

Figure 2. Correlations between production of hepatocyte-derived hemostatic proteins and total bile production during 6 hours of normothermic machine perfusion (NMP) of human donor livers using a plasma-free solution. Panel A; Factor II. Panel B; Factor V. Panel C; Factor VII. Panel D; Factor X Panel E; Fibrinogen. Panel F; Protein C. Panel G; Antithrombin III. Panel H; Plasminogen.